Dietary Inulin Increases Lactiplantibacillus plantarum Strain Lp900 Persistence in Rats Depending on the Dietary-Calcium Level

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ABSTRACT Synbiotics are food supplements that combine probiotics and prebiotics to synergistically elicit health benefits in the consumer. Lactiplantibacillus plantarum strains display high survival during transit through the mammalian gastrointestinal tract and were shown to have health-promoting properties. Growth on the fructose polysaccharide inulin is relatively uncommon in L. plantarum, and in this study we describe FosE, a plasmid-encoded β-fructosidase of L. plantarum strain Lp900 which has inulin-hydrolyzing properties. FosE contains an LPxTG-like motif involved in sortase-dependent cell wall anchoring but is also (partially) released in the culture supernatant. In addition, we examined the effect of diet supplementation with inulin on the intestinal persistence of Lp900 in adult male Wistar rats in diets with distinct calcium levels. Inulin supplementation in high-dietary-calcium diets significantly increased the intestinal persistence of L. plantarum Lp900, whereas this effect was not observed upon inulin supplementation of the low-calcium diet. Moreover, intestinal persistence of L. plantarum Lp900 was determined when provided as a probiotic (by itself) or as a synbiotic (i.e., in an inulin suspension) in rats that were fed unsupplemented diets containing the different calcium levels, revealing that the synbiotic administration increased bacterial survival and led to higher abundance of L. plantarum Lp900 in rats, particularly in a low-calcium-diet context. Our findings demonstrate that inulin supplementation can significantly enhance the intestinal delivery of L. plantarum Lp900 but that this effect strongly depends on calcium levels in the diet.

IMPORTANCE Synbiotics combine probiotics with prebiotics to synergistically elicit a health benefit in the consumer. Previous studies have shown that prebiotics can selectively stimulate the growth in the intestine of specific bacterial strains. In synbiotic supplementations the prebiotic constituent could increase the intestinal persistence and survival of accompanying probiotic strain(s) and/or modulate the endogenous host microbiota to contribute to the synergistic enhancement of the health-promoting effects of the synbiotic constituents. Our study establishes a profound effect of dietary-calcium-dependent inulin supplementation on the intestinal persistence of inulin-utilizing L. plantarum Lp900 in rats. We also show that in rats on a low-dietary-calcium regime, the survival and intestinal abundance of L. plantarum Lp900 are significantly increased by administering it as an inulin-containing synbiotic. This study demonstrates that prebiotics can enhance the intestinal delivery of specific probiotics and that the prebiotic effect is profoundly influenced by the calcium content of the diet.

KEYWORDS synbiotics, probiotics, prebiotics, Lactiplantibacillus plantarum, inulin, calcium, rats, intestinal persistence

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The concept of synbiotics was introduced in 1995 to describe a combination of probiotics and prebiotics that act in a synergistic manner to elicit health beneficial effects that are superior compared to the effects of the separately administered pre- or probiotic (1). Recently, the requirement for this synergy between both components has been emphasized by recommendation of the FAO (2). The mechanisms of synergy in a synbiotic can be achieved via different mechanisms, where the prebiotic compound could have a positive effect on survival and/or persistence of the administered probiotic and thereby enhance its health benefit, or the prebiotic could modulate the endogenous intestinal microbiota and thereby elicit a health benefit that enhances or amplifies the health benefit of the coadministered probiotic. It is important to note that these mechanisms are not mutually exclusive and could concurrently contribute to a health benefit of a synbiotic product (3).

Lactobacilli are carbohydrate-fermenting rod-shaped Gram-positive bacteria with a low G+C content that belong to the phylum Firmicutes and are a common member of the human gut microbial ecosystem (4). The genus, comprised of 261 species (as of March 2020), is phenotypically, ecologically, and genetically highly diverse, which has led to a recent taxonomic reclassification of the genus Lactobacillus into distinct clades with shared ecological and metabolic properties (5). Although the genus has been reclassified, many strains previously collectively classified as lactobacilli are marketed as probiotics (including Lactisaceibacillus rhamnosus, Limosilactobacillus reuteri, Lacticaseibacillus casei, Lactobacillus acidophilus, and Lactiplantibacillus plantarum) (6). L. plantarum is found in a wide variety of ecological habitats, including silage, fermented foods, and human and animal gastrointestinal (GI) tracts, and strains of this species display an impressive phenotypic diversity, especially in the context of strain-specific carbohydrate utilization (7, 8). Several L. plantarum strains were demonstrated to survive gastric transit and were detectable for 3 to 11 days postadministration in fecal samples (9, 10), and various health benefits of the consumption of L. plantarum have been reported (11–14). A recent landmark study reported a significant reduction of neonatal sepsis and mortality in newborn infants in rural India after synbiotic administration of fructo-oligosaccharides (FOS) and the probiotic L. plantarum ATCC 202195 (15). However, it remains unknown whether L. plantarum ATCC 202195 or the strains used in other synbiotic studies can actually utilize FOS as a substrate for growth. Moreover, separate pre- or probiotic interventions within the same population have not been performed, resulting in the fact that the mechanism(s) underlying the reported health benefit of synbiotic administration remains unclear. Utilization of FOS has been observed in L. plantarum reference strain WCFS1 up to a degree of polymerization (DP) of 3; however, utilization of longer-chain FOS (and inulin) has only been reported in L. plantarum strains isolated from pickled shallots and fermented fish of which the latter have shown to produce an extracellular inulin-hydrolyzing β-fructosidase that is involved in liberating fructose moieties from inulin (16–18).

Inulin consists of a series of linear β-2,1-linked fructose molecules with a terminal glucose that occur naturally in a multitude of vegetables and typically have a variable DP, ranging from 2 to 60 (denoted as GFn). FOS are derived from inulin through chemical or enzymatical conversion, and FOS consists of a mixture β-2,1-linked fructose molecules with or without terminal glucose moiety (two series of oligosaccharides denoted as GFn and Fn) with a DP ranging from 2 to 10 (19). Dietary FOS and inulin have been shown to have significant effects on the endogenous microbiota, and in particular their bifidogenic activity and stimulation of intestinal lactobacilli have been described both in animal models and in healthy human adults and infants (20–22). However, in addition to stimulatory effects on these benign microbes, dietary FOS supplementation has also been shown to increase enterobacterial populations in low-calcium regimes (21) and to increase the translocation and severity of Salmonella enterica serovar Enteritidis infection (23). The interplay between FOS and dietary calcium is relevant because in rat studies it was shown that high-calcium-phosphate diets can counteract the FOS stimulation of the enterobacteria and could prevent the FOS-induced increase in colonic permeability (24). Moreover, high calcium suppressed the severity
of orally induced salmonella infection in rats (21, 25) and decreased administered enterotoxigenic Escherichia coli (ETEC) colonization in rats (26). The latter effect was shown to translate to accelerated recovery from diarrhea elicited by an attenuated ETEC strain in an infection trial with healthy human volunteers. In addition, in human trials it was shown that higher intake of dietary calcium could ameliorate the negative effects associated with dietary FOS, which include an increased fecal mucin excretion and induced flatulence and intestinal bloating (22, 27). This illustrates a potential protective effect of dietary calcium phosphate against pathogenic enterobacterial infections that are undesirably facilitated (i.e., direct or indirect) by dietary FOS and inulin supplementation.

In this study, the previously reported inulin utilization by L. plantarum strain Lp900 (28) was further investigated at the molecular level. Heterologous expression experiments and high-performance anion-exchange chromatography (HPAEC) analyses confirmed that L. plantarum Lp900 grown on inulin media produces an extracellular β-fructosidase. The identified β-fructosidase effectively hydrolyzes the substrate, and this is the only function required for inulin utilization in L. plantarum. Furthermore, the impact of inulin as a dietary ingredient or as a coadministered (synbiotic) nutrient on the intestinal persistence of L. plantarum Lp900 was investigated in Wistar rats, using diets that varied in dietary calcium concentrations.

**RESULTS**

**Supernatant of Lp900 grown on inulin stimulates growth of strain WCFS1 on inulin.** Reference strain L. plantarum WCFS1 was employed as a negative control for the utilization of DP > 3 inulin (16) to demonstrate the inulin-utilizing capacities of L. plantarum Lp900. L. plantarum Lp900 was grown on 2-fold-diluted de Man, Rogosa, and Sharpe medium (1/2MRS) without the addition of a carbon source (1/2MRS-C) supplemented with 0.5% inulin or glucose to early stationary phase (optical densities at 600 nm [OD600s] = 2.63 and 3.08, respectively), and spent culture supernatant was filter sterilized and added (25% [vol/vol]) to 1/2MRS-C supplemented with 0.5% inulin or glucose. L. plantarum WCFS1 was inoculated in these media and grown overnight at 37°C. WCFS1 grown on 1/2MRS-C supplemented with 0.5% inulin or glucose without the addition of spent culture supernatant of Lp900 grew to OD600s of 0.69 and 2.80, respectively (Fig. 1A). The addition of spent culture supernatant of Lp900 grown on glucose did not affect growth of WCFS1 on 1/2MRS-C supplemented with 0.5% inulin (OD600 of 0.69). Importantly, the addition of spent culture supernatant of L. plantarum Lp900 grown on 1/2MRS-C supplemented with 0.5% inulin stimulated the growth of L. plantarum WCFS1 1/2MRS-C supplemented with 0.5% inulin to reach a final OD600 of 1.44. This increase in growth is not reaching the level of growth of WCFS1 on glucose, suggesting that only part of the inulin substrate is degraded during overnight incubation with the spent culture supernatant of Lp900.

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis of the inulin substrate detected degrees of polymerization up to DP 40 (Fig. 1B). HPAEC-PAD elution patterns of spent culture supernatants revealed the complete depletion of the inulin substrate by overnight growth of the L. plantarum Lp900, whereas strain WCFS1 only depleted the glucose, fructose, sucrose, and 1-kestose fraction of the inulin substrate (Fig. 1C). The latter observation is in agreement with previously reported utilization of only short-chain FOS (i.e., up to a DP of 3) by L. plantarum WCFS1 (16). When exposed to 25% of spent culture supernatant of L. plantarum Lp900 grown on inulin, a clear degradation of inulin was observed, although the substrate was not completely degraded after overnight incubation with the spent culture supernatant (see Fig. S2 in the supplementary material). The degradation of inulin coincided with increased levels of fructose and 1-kestose, but not sucrose (see Fig. S2), which is in contrast to previous reports using culture supernatants of inulin utilizing Lactcaseibacillus paracasei strain 1195 in which inulin hydrolysis primarily generated fructose and no oligomeric intermediates (29). Moreover, the partial degradation of inulin by spent culture supernatant of L. plantarum Lp900 into fructose
and 1-kestose explains the observed partial growth stimulation of *L. plantarum* WCFS1 (Fig. 1D). The results indicate that *L. plantarum* Lp900 excretes an inulin-hydrolyzing enzyme that liberates fructose and 1-kestose from the long-chain polymers present in the inulin substrate (see Fig. S2). Notably, no inulin degradation was observed when exposing this substrate to spent culture supernatant of *L. plantarum* Lp900 grown on glucose (see Fig. S2), indicating that the production of this enzyme by *L. plantarum* Lp900 is controlled by growth on inulin and corroborating that the addition of this spent culture supernatant could not stimulate growth of *L. plantarum* WCFS1 on inulin-containing media.

To further investigate the presence of such secreted enzyme, spent culture supernatants of Lp900 grown on 1/2MRS-C supplemented with 0.5% inulin or glucose were desalted and analyzed by SDS-PAGE, followed by silver staining. The spent culture supernatants of inulin-grown *L. plantarum* Lp900 contained a protein with an apparent molecular weight of \( \sim 140 \text{kDa} \) that was absent in the spent culture supernatant of *L. plantarum* Lp900 grown on glucose (see Fig. S2), indicating that the production of this enzyme by *L. plantarum* Lp900 is controlled by growth on inulin and corroborating that the addition of this spent culture supernatant could not stimulate growth of *L. plantarum* WCFS1 on inulin-containing media.

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**In silico analysis of *L. plantarum* strain Lp900-encoded \( \beta \)-fructosidase.** *L. plantarum* strain Lp900 has a genome of 3.2 MB and harbors six plasmids. One of these plasmids (48.8 kb in size) encodes a so-called "FOS operon" (17) containing the fosE-encoded \( \beta \)-fructosidase and genes encoding a phosphotransferase system that is predicted to import fructose (designated *fosABCDX*) and a transcriptional regulator (designated *fosR*) (J. Fuhrén, R. Nijland, M. Wels, J. Boekhorst, and M. Kleerebezem, unpublished data). Although previous research concluded that there were no
indications that this FOS operon was encoded on a plasmid in *L. plantarum* strains P14 and P76 (17), it is plasmid encoded in strain Lp900. Moreover, proteins with significant identity to the *fosE*-encoded enzyme (approximately 60 to 80% identity with virtually full-length alignment) were identified in various *L. plantarum* strains, as well as in related species such as *Pediococcus acidilactici* and *P. pentosaceus*, and most of these fructosidases were annotated to be encoded by plasmids of varying sizes (data not shown). In contrast, homologs of the *fosE*-encoded β-fructosidase in *Lacticaseibacillus casei* and *Lacticaseibacillus paracasei* strains appear to be encoded on the chromosomes of these strains, which is in agreement with previous reports (29, 30). The various β-fructosidases identified in these different species consistently contain two conserved glycosyl hydrolase family 32 (GH32) domains consisting of an N-terminal 5-fold β-propeller, which functions as the active catalytic site, and a C-terminal domain, which consists of two six-stranded β-sheets forming a sandwich-like fold, and is proposed to be involved in carbohydrate recognition (31). There are minor strain-specific amino acid substitutions throughout the GH32 domains, especially in the protein encoded by *L. plantarum* Lp900, but in all the protein sequences the N-terminal domain contains the highly conserved catalytic residues (see Fig. S4). In addition, all encoded proteins appear to contain a signal peptide and a LPxTG-like domain, which is in line with earlier observations (17), and underpins the extracellular peptidoglycan anchoring of the β-fructosidase encoded by *L. plantarum* Lp900 (see Fig. S4), although our experimental observations clearly demonstrate that at least part of the β-fructosidase produced in strain Lp900 is released into the environment and is not covalently bound to the cell wall.

The *L. plantarum* Lp900-encoded β-fructosidase encompasses 1,148 amino acid residues, and the predicted molecular weight of the mature protein (i.e., after removal of the signal peptide and cleavage of the LPxTG motif by sortase A) is 114 kDa, which is lower than the apparent molecular weight of the protein detected in the inulin-grown culture supernatant (Fig. 2). The Lp900-encoded β-fructosidase protein sequence was compared to homologous proteins identified in *L. plantarum*, *P. acidilactici*, *P. pentosaceus*, *Lacticaseibacillus paracasei*, and *Lacticaseibacillus casei* using the maximum-likelihood method. In this analysis, a chromosomally encoded GH32 domain containing levansucrase from *Lactococcus piscium* MKFS47 (32) with an approximate 42% identity with the FosE of *L. plantarum* Lp900 was used as an outgroup. β-Fructosidases derived from *Lacticaseibacillus paracasei* and *L. plantarum* (with exception of strain Lp900) were grouped into two clusters of highly homologous proteins (within-cluster identity levels of 99 to 100%) that were distinct and species specific (identity level of approximately 64% between clusters) (Fig. 3). Notably, the β-fructosidase encoded by *L. plantarum*

**FIG 2** SDS-PAGE and silver staining of near stationary spent culture supernatants of Lp900. Results for desalted spent culture supernatants of Lp900 grown on glucose (lane B) or inulin (lane C) and Spectra multicolor high-range protein ladder (lane A) are shown. The arrow indicates a protein band that is uniquely present in spent culture supernatant of Lp900 grown on inulin and not in glucose with an estimated molecular weight of 140 kDa.
Lp900 displayed the highest identity with a protein encoded by Lacticaseibacillus casei ATCC 334 (75%), and these two enzymes were more closely related to β-fructosidases found in the pediococci (73 and 88% identities, respectively) than those canonically found in L. plantarum or Lacticaseibacillus paracasei, suggesting independent evolutionary acquisition by horizontal gene transfer (potentially from a pediococcal species) in Lp900 and ATCC 334 (Fig. 3).

Heterologous expression of Lp900-derived β-fructosidase in L. plantarum strain NC8 results in utilization of inulin for growth. To unambiguously establish the inulin hydrolyzing function of the predicted β-fructosidase of L. plantarum Lp900, its encoding gene fosE was cloned under the control of a constitutive lactococcal bacteriophage φISKIIIG promoter (33) in expression vector pNZ278 (33). The resulting pNZ278-fosE was transformed to plasmid-free L. plantarum NC8 that is not able to degrade and utilize inulin (final OD₆₀₀ of ~0.49 on inulin-supplemented 1/2MRS-C), whereas its pNZ278-fosE transformant could effectively utilize inulin, reaching a final culture density of 3.02 (OD₆₀₀) in 1/2MRS-C supplemented with 0.5% inulin (Fig. 4A). This result demonstrates that the fosE-encoded function is the only function required to support growth on inulin and underpins that the genetically linked functions within the FOS operon are not required for this phenotype. Moreover, HPAEC analysis of spent culture supernatants of pNZ278-fosE-harboring NC8 confirmed the complete depletion of the inulin substrate, whereas the original NC8 strain utilized only the fructose, sucrose, and 1-kestose fractions of the substrate (Fig. 4B).

Dietary inulin increases Lp900 persistence in rats in a high-calcium diet. Eight-week-old male Wistar (n = 8 per group) rats were acclimatized to diets based on American Institute of Nutrition (AIN) standards (66), supplemented with inulin or cellulose with high or low calcium for 2 weeks; each rat was then gavaged with a standardized dose of Lp900-R. No differences were observed in the food intake and growth of the animals on the different diets postgavage (data not shown). Notably, the consistency of the feces differed between the groups that were fed the different diets. Based on visual observation, fecal material from the high- and low-calcium control groups (i.e., Hca and Lca) appeared dryer compared to fecal material from the inulin-
supplemented groups (i.e., HcaInu and LcaInu), which was not unexpected as the presence of inulin in the diet is anticipated to have an effect on the water content in the feces (34). Dry/wet ratios of fecal samples obtained from each group confirmed a significantly increased water content for the LcaInu group compared to the Lca group ($P = 0.0002$), but this effect of inulin was only a trend when comparing the HcaInu group to the Hca group ($P = 0.0738$) (see Fig. S5 in the supplementary material).

Feces were freshly collected in the morning at baseline (prior to gavage with *L. plantarum*) and each morning after intragastric gavage. The average recovery of Lp900-R on day 1 postgavage was relatively similar for all groups and ranged between $1.7 \times 10^7$ and $6.9 \times 10^7$ CFU per g of feces (wet weight). In rats that were fed the Lca and Hca diets, the recovery of *L. plantarum* Lp900-R from feces rapidly declined to an average of $2.3 \times 10^5$, and $9.1 \times 10^3$ per g of feces (wet weight) on day 3 postgavage, respectively. In particular in the group of rats on the HcaInu diet the *L. plantarum* intestinal persistence was significantly higher compared to the related, nonsupplemented (i.e., cellulose supplemented) diet, which is illustrated by the 20- to 200-fold higher average number of CFU recovery on days 2 to 5 postgavage and 5- to 10-fold higher CFU recovery on subsequent days (Fig. 5A and B). A more moderate and trending higher persistence was also observed in the LcaInu diet compared to the nonsupplemented Lca diet, illustrated by the ~7-fold higher average CFU recovery on days 2 and 3 postgavage. However, the increased recovery of Lp900 in the Lcalnu group compared to the Lca group was not considered statistically significant ($P = 0.094$ and $P = 0.130$ for days 2 and 3, respectively), which may be due to relatively high-level variation in CFU recovery between the rats in these groups (see Fig. S5). Linear decline rates were calculated for the time frame in which the majority of the samples allowed the recovery of Lp900-R (i.e., the first 5 days postgavage) to quantify the rate of persistence decline in individual rats. Comparison of the linear decline rates confirm the significant increased persistence of the HcaInu group compared to the Hca group, whereas the linear decline rates of the Lcalnu group were not statistically different from the Lca group (Fig. 6A and B). However, the linear decline rates for rats in the inulin-supplemented diet groups displayed substantial rat-specific variation, which reflects the high rat-specific variation seen in *L. plantarum* Lp900 CFU recovery in these groups (Fig. 6A and B). The dry-weight/wet-weight ratios of the fecal samples differed slightly in the different diet groups (see Fig. S5) and, taking dry-weight rather than wet-weight corrections of the recovery of *L. plantarum* from fecal samples, identified a significant increase in
recovery on day 2 in fecal samples from rats fed on the LcaInu diet compared to those fed the Lca diet, which was considered not significant using wet-weight corrections (data not shown). However, the overall conclusions of the persistence measurements or the comparative analysis of the abundance-decline rate estimations are not impacted by this observation. Thereby, these results robustly demonstrate that the persistence of *L. plantarum* Lp900-R is influenced by the diet regimen of the rats and is especially enhanced by the inulin supplementation in the high-calcium diet.

**Synbiotic inulin and Lp900 administration increases survival and short-term persistence in the rat intestine.** In a similar experiment, rats adapted to the AIN diet with low or high calcium levels received either a probiotic gavage containing *L. planta-**

![Diagram](A.png) **FIG 5** Persistence over time of intragastrically administered Lp900 in adult male rats. (A) Persistence of Lp900-R in rats on Lca (open circles) and Lcalnu (filled circles) diets after a single gavage with approximately $8 \times 10^6$ CFU of *L. plantarum* Lp900-R on day 0. (B) Persistence of Lp900-R in rats on Hca (open circles) and Hcalnu (filled circles) diets after a single gavage with approximately $8 \times 10^6$ CFU of *L. plantarum* Lp900-R on day 0. (C) Persistence of Lp900-R in rats on Lca diets receiving a probiotic (LcaP; open circles) or synbiotic (LcaS; filled circles) (9 x 10^9 CFU of Lp900-R in a 27.5% [wt/vol] Orafti GR inulin solution). (D) Persistence of Lp900-R in rats on Hca diets receiving a probiotic (HcaP; open circles) or synbiotic (HcaS; filled circles) (9 x 10^9 CFU of Lp900-R in a 27.5% [wt/vol] Orafti GR inulin solution). All CFU counts are log transformed and expressed as means ± the SEM (n=8 per diet group). Asterisks indicate at which time points the inulin supplemented (either by diet or gavage) are significantly different than the respective control group as detected by a two-sided nonparametric Mann-Whitney U test with $\alpha = 0.05$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

![Diagram](B.png) **FIG 6** Linear decline rates of modeled Lp900 persistence curves in adult male rats. Decline rates of linear models fitted to log$_{10}$ converted recovered Lp900-R in rats for the first 5 days postgavage. (A) Hca and Hcalnu diets; (B) Lca and Lcalnu diets; (C) HcaP and HcaS diets; (D) LcaP and LcaS diets. Asterisks indicate whether the inulin supplemented (either by diet or gavage) are significantly different than the respective control group as detected by a two-sided nonparametric Mann-Whitney U test with $\alpha = 0.05$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).
rum Lp900-R or a synbiotic gavage containing *L. plantarum* Lp900-R suspended in an inulin solution. No differences were observed in food intake and growth of the animals on the different diets postgavage (data not shown).

Intestinal persistence of *L. plantarum* Lp900-R over time was assessed as described above. The average recoveries at 1 day postgavage were $3.7 \times 10^6$ and $1.7 \times 10^7$ CFU per g of wet feces in the LcaP and HcaP groups, respectively, and both groups displayed a similar decline of Lp900-R recovery. The synbiotic groups (LcaS and HcaS) showed an initial higher recovery of approximately $8 \times 10^6$ CFU per g of wet feces but displayed a decline rate of Lp900-R recovery that was very similar to that observed in the probiotic groups. *L. plantarum* Lp900-R recovery was 10- to 100-fold higher in the LcaS group compared to the LcaP group during days 2 and 3 postgavage, and the difference was considered statistically significant from days 1 to 5 postgavage (Fig. 5C and D). A more modest increased recovery of *L. plantarum* Lp900 (6- to 7-fold) was observed in the HcaS compared to the HcaP group, which was considered statistically significantly different on days 1 and 3, and trending on day 2 ($P = 0.06$) (Fig. 5C and D). Estimations of linear decline rates in the individual rats in this experiment revealed that all groups exhibit comparable decline rates and that neither diet nor the format in which Lp900 administration was performed (i.e., probiotic or synbiotic) had a detectable impact on the rate of population decline in these groups (Fig. 6C and D).

Taken together, these results indicate that initial survival and persistence of *L. plantarum* Lp900-R in the rat intestinal tract are significantly enhanced by coadministration of inulin. This effect appeared largely independent of the calcium levels in the diet, although it appeared somewhat larger with the low-calcium diet. The initial survival and/or persistence resulting from this synbiotic coadministration has a lasting effect in increased abundance of *L. plantarum* Lp900-R on consecutive days, although the population decline rate appears to be unaffected by the substrate coadministration.

**Short-term local abundance of *L. plantarum* strain Lp900 is unaffected by dietary regime or by substrate coadministration.** After the second gavage, no differences in the recovery of *L. plantarum* Lp900-R from the distal ileum or midcolon lumen at either time point were observed irrespective of the diet regime or administration modus employed (see Fig. S7 in the supplementary material), indicating that inulin provided as a diet ingredient or as a coadministered substrate (i.e., synbiotic) was unable to increase short-term local persistence and/or abundance of *L. plantarum* Lp900-R in the small (distal ileum) or large (midcolon) intestine.

**DISCUSSION**

Symbiotics aim to combine probiotics and prebiotics to synergistically elicit their health effects. The prebiotic compound can contribute to the health benefits either by directly stimulating the viability, activity, and/or persistence of the coadministered probiotic and/or by stimulation of specific members of the endogenous microbiota, thereby promoting health benefits in parallel to those elicited by the probiotic component (3).

In this study we investigated the inulin utilization capacity of *L. plantarum* at a phenotypic and molecular level using the strain Lp900 that we previously identified as an inulin- and FOS-utilizing strain that completely digests these substrates and is able to grow equally efficiently on these substrates compared to glucose (28). This phenotype was in agreement with the presence of a plasmid-encoded fos operon in strain Lp900 (Fuhren et al., unpublished), which has previously described to play a role in the utilization of fructo-polysaccharides in *L. plantarum* (17). Moreover, our results establish that only the extracellular $\beta$-fructosidase (FosE) encoded within this operon is required to facilitate inulin utilization in *L. plantarum*. The FosE inulin-hydrolyzing activity appeared to be partially released in the Lp900 culture supernatant, which is not uncommon for LPxTG-anchored $\beta$-fructosidases (17, 32, 35), although the subcellular localizations may be species (and possibly strain) specific. For example, the $\beta$-fructosidase of *Lactcaseibacillus paracasei* 1195 was reported to be cell wall bound and absent from the culture supernatants (29), whereas the FosE homologue of *Lactcaseibacillus casei* IAM1045 (designated LevH1) was predominantly found in the culture supernatant (32).
The efficiency of cell wall anchoring of these LPxTG proteins may depend on sequence specificity and efficiency of the sortase A of the host organism, as has been described for *Staphylococcus aureus* (36). Notably, the anchor sequence for FosE in Lp900 (LPNTG) deviates from the consensus sortase motif of *L. plantarum* (LPQTxE [37]), which may reflect the proposed acquisition of this fosE gene through horizontal gene transfer from a different species. Intriguingly, sequence comparison of the FosE of *L. plantarum* Lp900 revealed its distinction from other members of this enzyme family present in *L. plantarum* and *Lacticaseibacillus paracasei*, supporting its independent acquisition in strain Lp900 by horizontal gene transfer, most likely from a pediococcal species where this function was also described to be plasmid encoded.

Alignments of the *L. plantarum*, *Lacticaseibacillus paracasei*, *Lacticaseibacillus casei*, and pediococcal β-fructosidases revealed considerable variability in protein length and sequence but confirmed the consistent presence of two GH32 domains and its associated sequence motifs (WMNDPNG, RDP, and ECP) that encompass the “catalytic triad” in this enzyme family (31). Most of the variation between strains and species is due to N-terminal repeat stretches and a variable number of C-terminal Big3 (*Lacticaseibacillus paracasei* and *Lacticaseibacillus casei*) or MucBP (*L. plantarum* and pediococcal strains) domains, the latter of which are suggested to play a role in substrate adherence in the GI tract (38, 39). Despite its overall sequence conservation, amino acid substitutions within the GH32 domains were particularly prevalent in the *L. plantarum* Lp900 FosE (30 unique residues; see Fig. S4 in the supplemental material). These characteristics are in good agreement with the observation that *L. plantarum* Lp900 FosE clusters in a clade that is separate from the homologues found in other *L. plantarum* or *Lacticaseibacillus paracasei* strains and is more closely related to pediococcal FosE-like enzymes. Interestingly, most of the FosE proteins of *L. plantarum* that are available in the databases derive from strains isolated in Southeast Asia or Australia (17), including isolates from fermenting (shell-)fish or vegetables that traditionally occur spontaneously and involve “autochthonous” environmental microorganisms (40). In contrast, *L. plantarum* Lp900 originates from ogi, a Nigerian red-sorghum-based porridge which is also fermented by spontaneously occurring microorganisms (41), suggesting that the separate clustering of its FosE homologue relates to its geographical origin, which implies that characterization of other *L. plantarum* isolates originating from African fermented products may reveal additional β-fructosidases that are more related to the enzyme found in strain Lp900.

In the first animal trial performed in this study, dietary inulin profoundly elevated and extended the intestinal persistence of gastrically gavaged Lp900 in a high-calcium-diet background. In contrast, Lp900 fecal abundance decreased rapidly in rats fed a high-calcium control diet that was not supplemented with inulin, leading to barely detectable fecal levels after 4 days. This impact of inulin supplementation was much less pronounced in a low-calcium diet context. Similar results were reported for *L. plantarum* strain no.14, which displayed increased intestinal persistence in mice that were fed an inulin-supplemented AIN-93 diet, and it should be noted that the standard AIN-93 diet contains calcium levels comparable to the high calcium level used in our study. However, the increased level of persistence in that study was short-lived (until ~30 h postgavage) and *L. plantarum* no.14 was no longer detectable after 48 h, irrespective of inulin supplementation of the diet (18). The effect size in our study is much larger, since *L. plantarum* Lp900 could be readily detected until 9 days postgavage in fecal samples from rats that were fed an inulin-supplemented high-calcium diet. These persistence differences may in part be explained by the different animal models used and/or differences in the inherent intestinal fitness of the two *L. plantarum* strains used. Our results suggest that inulin utilization by *L. plantarum* Lp900 underlies the increased intestinal persistence in inulin-supplemented high-calcium diets. However, our results cannot exclude that the changes in *L. plantarum* Lp900 persistence occur independently of the strain’s capacity to utilize inulin as a substrate for growth and are
attributable to other environment-modulating effects of dietary inulin that favor \textit{L. plantarum} colonization, irrespective of its capacity to utilize inulin.

Our experiments also show that the coadministration of \textit{L. plantarum} Lp900 with inulin as a substrate (synbiotic) can enhance the initial survival and persistence of the bacteria in the intestinal tracts of rats fed a high- or low-calcium diet. Although this enhancement of \textit{L. plantarum} abundance persisted for several days, the synbiotic administration did not appear to affect the decline rate in intestinal abundance of \textit{L. plantarum}. This is in apparent contrast to the effects observed when inulin is present as a continuous supplement in the rat diet, indicating that reduced decline rates depend on the continuous presence of the substrate rather than a single bolus of coadministered substrate, which would agree with persistence stimulation by dietary inulin depending on the inulin utilization capacity of \textit{L. plantarum} Lp900. The initial enhancement of survival and/or persistence in the substrate coadministration regime could depend predominantly on the substrate-dependent energy state of the bacterial culture that may increase survival of intestine associated stress conditions (e.g., stomach acid and small intestinal bile acid exposure). Notably, such substrate-dependent survival has been reported for \textit{L. plantarum} and several other species using an \textit{in vitro} model that mimics gastrointestinal conditions (42). Similar to our results, \textit{L. plantarum} no.14 also displayed strongly increased short-term survival when coadministered with inulin in mice that were fed a high-fat diet (43). However, survival at later time points postadministration were not reported for \textit{L. plantarum} no.14, and previous studies have reported that \textit{L. plantarum} displays reduced intestinal colonization in mice fed a high-fat diet (44), disallowing a more complete comparison of the results between the mouse model study and the work presented here.

The importance of dietary calcium levels in the context of the efficacy of inulin supplementation in stimulating the \textit{L. plantarum} Lp900 intestinal persistence is found in the extrapolation of these variations in calcium levels to common human diets. In general, calcium intake levels are relatively low in many countries and are commonly lower in women than in men (45), especially in many Asian, African, and South American countries calcium intake is estimated to be between 400 to 700 mg/day or even lower (45), which may result from lactose malabsorption prevalence in these populations (46). In contrast, an average calcium intake above 1000 mg/day has been reported in northern European countries (45). The low- and high-calcium regimes used here can be extrapolated to daily calcium intake levels in human populations of approximately 600 and 2,000 mg per day, respectively (24). Thereby, our low-calcium regime reflects habitual dietary intake in a large part of the human population, while the high-calcium regime reflects the upper boundaries of the habitual intake in populations that consume substantial amounts of calcium-rich dairy products (47). In addition, the 40-g/kg supplementation of inulin in the experimental diets would correspond to 20 g/day in human diets (based on a daily 500-g dry-weight food intake), which is also not un conceivable considering the recommended dietary fiber intake of 25 g/day (48). Besides the general health benefits associated with calcium (i.e., bone health), high-dietary-calcium regimes were shown in rat studies to counteract inulin- and FOS-induced entero-bacteria stimulation while concurrently stimulating the endogenous lactobacilli (21), resulting in decreased severity of serovar Enteritidis and ETEC infections in these models (25, 26). It has been suggested that calcium forms complexes with phosphate that can dissolve in the GI tract, thereby increasing the buffering capacity of the intestinal lumen and the precipitation of cytotoxic surfactants, e.g., secondary bile acids (49, 50). Since such surfactants are especially detrimental for Gram-positive bacteria (51), their precipitation by high calcium levels may contribute to the higher persistence of \textit{L. plantarum} Lp900 in inulin supplemented high-calcium diets.

The importance of better understanding the relationship between pre- and probiotics in synbiotic supplements is driven by the health benefits reported for these supplements. A landmark study in this area demonstrated decreased neonatal sepsis and mortality in newborns in rural India upon administration of a synbiotic containing \textit{L.}}
plantarum ATCC 202195 and FOS (15). Unfortunately, it is not known whether this effect was dependent on the probiotic supplement or could also be achieved with supplementation of either its probiotic or prebiotic constituent. In addition, it was not reported whether L. plantarum ATCC 202195 is able to efficiently utilize FOS as a substrate for growth. However, the absence of a fosE-like gene (data not shown) in the draft genome of L. plantarum ATCC 202195 (GenBank accession no. GCA_004354995.1) indicates that this strain is probably unable to utilize the FOS substrate beyond its fructose, sucrose and possibly 1-kestose “contaminants.” This implies that the reported health effects do not depend on growth stimulation of the probiotic by the coadministered prebiotic and suggests that the constituents of this synbiotic act independently, provided that their synergy is indeed a requirement to elicit the observed health impact. Similarly, in various studies that report health effects of synbionts containing L. plantarum and FOS or inulin (52–54) it remains unknown whether the L. plantarum strains used can utilize the prebiotic substrate for growth, which leaves the mechanism underlying their synergistic effect on health to be determined. Our study suggests that the capacity to utilize inulin underlies the enhanced intestinal persistence of L. plantarum Lp900, which would support that enhanced in situ delivery of the probiotic by coadministration of a prebiotic that supports its growth could explain the synergistic effects of synbiotic supplementation. Moreover, our study also exemplifies the notion that the efficacy of health benefits associated with pre-, pro-, or synbionts may be strongly influenced by other dietary factors, analogous to the profound role of dietary calcium levels demonstrated in this study.

MATERIALS AND METHODS

Culturing of strains and collection of supernatants. L. plantarum strain Lp900 was inoculated in 2-fold diluted de Man, Rogosa, and Sharpe medium (1/2MRS) without the addition of a carbon source (1/2MRS-C) or supplemented with either 0.5% Orafti GR inulin (BENEO-Orafti, Oreye, Belgium) or 0.5% glucose and grown overnight at 37°C. Overnight cultures were subcultured (1:100) in the same media, and growth was monitored by determining of the OD600 with a SpectraMax M5 microplate spectrophotometer (Molecular Devices, Jan Jose, CA). Cultures were collected after 7.5 h, at OD600s of 2.63 and 3.08, from inulin- and glucose-supplemented media, respectively. Culture supernatants were collected by removal of the cells by centrifugation (4,000 × g, 10 min, 4°C), pH neutralized by titration with 2 M sodium hydroxide to pH 6.8, and filter sterilized through a 0.2-μm Whatman cellulose acetate filter and subsequently used as a medium supplement (see below).

L. plantarum strain WCFS1 was grown overnight at 37°C in 1/2MRS-C supplemented with 0.5% glucose and subsequently subcultured (1:100) in the same medium, and cultures were collected after 7.5 h at an OD600 of 2.98 and subcultured (1:500) in 1/2MRS-C supplemented with 0.5% inulin that was mixed with spent culture supernatants (25% [vol/vol]) of L. plantarum Lp900 grown on 1/2MRS-C medium supplemented with either inulin or glucose. After overnight growth at 37°C, the final OD600s of these cultures were determined. In parallel, the same spent culture supernatants were also added (25% [vol/vol]) to noninoculated (i.e., sterile) 1/2MRS-C supplemented with 0.5% inulin, followed by incubation overnight at 37°C to assess inulin digestion by HPACE (see below).

High-performance anion-exchange chromatography. Media and spent culture supernatants were diluted 1:20 in MilliQ and were analyzed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). An IC5000 HPAEC ( Dionex, Sunnyvale, CA) was used with CarboPac PA1 column (2 × 250 mm) in combination with CarboPac PA1 guard column (2 × 50 mm). Eluents (0.1 M sodium hydroxide) and B (1 M sodium acetate in 0.1 M sodium hydroxide) were used at a flow rate of 0.3 ml/min in a linear gradient from 0 to 40% B in 40 min, followed by column washing with 100% B (5 min) and reequilibration of the column by 100% A (15 min). The elution of inulin-derived oligosaccharides was monitored using a pulsed amperometric detector (Dionex ICS-5000 ED). The column oven was kept at 20°C, and the sample tray was set at 10°C. Elution profiles were visualized and analyzed using Chromelon V7.2 (Thermo Fisher Scientific, Waltham, MA) software.

SDS-PAGE of spent culture supernatants. Spent culture supernatants of L. plantarum Lp900 growth on 1/2MRS-C supplemented with 0.5% inulin or 0.5% glucose (OD600s of 3.3 and 3.1, respectively) were desalted with 7K MKWCO Zeba spin desalting columns (Thermo Scientific, Waltham, MA) and mixed with an equal volume of 2 × SDS loading buffer, followed by incubation at 95°C for 10 min. Samples (20 μl) were loaded onto an 10% Mini-Protean TGX precast gel that were run 10 min at 40 V, followed by 80 min at 100 V, and silver stained using a SilverXpress silver staining kit (Invitrogen/Thermo Scientific, Waltham, MA), according to the manufacturer’s protocol. A portion (10 μl) of Spectra multicolor high-range protein ladder (Thermo Scientific, Waltham, MA) was used as a molecular weight reference.

In silico analysis of β-fructosidas. The protein sequence of the beta-fructosidase encoded by L. plantarum Lp900 (FosE; accession numbers CP059168 to CP059174 [Fuhren et al., unpublished]) was used to retrieve related β-fructosidas from the public sequence database of the National Center for
Biotechnology Information (NCBI) (55, 56) using BLASTP (57). Protein sequences were functionally annotated using InterProScan 5 (58) and the CaZy database (59), and the subcellular localization was predicted using SignalP-5.0 (60). Protein sequences were aligned using Muscle (61), visualizing the alignments with Jalview v.2.11.0 (62), estimating the maximum likelihood with RAxML v.8.0.0 (63), and predicted using SignalP-5.0 (60). Protein sequences were aligned using Muscle (61), visualizing the alignments with Jalview v.2.11.0 (62), and constructing phylogenetic trees with FigTree v.1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

**TABLE 1**  
<table>
<thead>
<tr>
<th>Strain, plasmid, or primer</th>
<th>Relevant feature(s) or sequence (5′–3′)</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. plantarum</em> WCFS1</td>
<td>Single-colony isolate of <em>L. plantarum</em> NCIMB8826</td>
<td>68</td>
</tr>
<tr>
<td><em>L. plantarum</em> Lp900</td>
<td>Harbors six plasmids, one of which encodes an “FOS operon,” including a β-fructosidase</td>
<td>Fuhren et al., unpublished</td>
</tr>
<tr>
<td><em>L. plantarum</em> Lp900-R</td>
<td>Rifampin-resistant derivative of Lp900</td>
<td>This study</td>
</tr>
<tr>
<td><em>L. plantarum</em> NC8</td>
<td>Plasmid-free strain of <em>L. plantarum</em></td>
<td>69</td>
</tr>
<tr>
<td><em>L. lactis</em> NZ9000</td>
<td>MG1263 derivative harboring nisR integrated in its genome</td>
<td>70</td>
</tr>
<tr>
<td><em>L. lactis</em> MG1363</td>
<td>NCD0712-derived strain that lacks plasmids and prophages</td>
<td>71</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pNZ278</td>
<td>Cm'; containing gusA under control of the bacteriophage φSK11G promoter</td>
<td>33</td>
</tr>
<tr>
<td>pNZ278-fosE</td>
<td>Cm'; pNZ278 derivative in which the gusA coding region is replaced by <em>L. plantarum</em> Lp900 fosE</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Primers</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1</td>
<td>GAGGACGCGTTCTAAGTCGTATCCGTGGAATAGATTCCA</td>
<td>This study</td>
</tr>
<tr>
<td>S2</td>
<td>AACATGCTGAAGAGCATCTCATCTCAGTGAAATACCCAC</td>
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</tr>
<tr>
<td>S3</td>
<td>GTGAGCGTTTTACCACCGCAGATGCTCTCAGTACAGTT</td>
<td>This study</td>
</tr>
<tr>
<td>S4</td>
<td>TGGGATCATTTCTACACCGGATACGACTTAGAACCGTCCT</td>
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</tr>
<tr>
<td>S5</td>
<td>CCTTAAACGAGGTTGCTGTTA</td>
<td>This study</td>
</tr>
<tr>
<td>S6</td>
<td>GCTTCTACGTGAAGATTGCC</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Animal experiments and study design.** A rifampin-resistant derivative of *L. plantarum* Lp900 was selected to enable its selective recovery from fecal samples. To this end, *L. plantarum* Lp900 was precultured in 1/2MRS-C supplemented with 0.5% inulin, plated (100 µl) on MRS agar containing rifampin (100 µg/ml), and incubated at 37°C for 2 days. Single-colony isolates of rifampin-resistant *L. plantarum* Lp900 were verified for their capacity to grow on 1/2MRS-C supplemented with 0.5% inulin and containing 100 µg/ml rifampin, while they also displayed unimpaired growth on regular MRS, leading to the isolation of rifampin-resistant *L. plantarum* Lp900-R. The Lp900-R cultures used in the animal experiments were grown overnight in 1/2MRS-C supplemented with 100 µg/ml rifampin and 0.5% inulin, followed by harvesting the cells by centrifugation to subsequently suspend the pellet in phosphate-buffered saline solution (PBS) at the density required for the intragastric gavages (see below). Two separate animal experiments were performed, evaluating either *L. plantarum* Lp900-R persistence in inulin supplemented diets in high- and low-dietary-calcium contexts (Fig. 7A) or the persistence of *L. plantarum* Lp900-R when administered as probiotic or symbiotic gavage (Fig. 7B). Eight-week-old specific-pathogen-free male Wistar rats (Harlan, Horst, The Netherlands), with an average body weight of 325 g, were housed individually in standard rodent cages in a climate-controlled room kept at 22 to 24°C and 50 to 60%
humidity and under a 12-h light-dark cycle. During both trials, rats received food and water ad libitum and were weighed twice a week, and food intake was monitored daily.

Animals (n = 8 per diet group) were fed a powdered AIN-93-derived diet (66) with a high-fat content (200 g of fat/kg), mimicking the composition of a Western human diet, to evaluate the effect of dietary inulin on the persistence of \textit{L. plantarum} Lp900-R in a dietary calcium context (Fig. 7A). The diets (Table 2) were supplemented with either 40 g/kg cellulose (Arbocel type B899; JRS, Zutphen, The Netherlands) or 40 g/kg Orafti GR inulin and contained either 30 or 100 mmol/kg (4.08 or 13.60 g/kg) CaHPO$_4$ (Acros Organics, Thermo Scientific); diets were designated designated as low calcium (Lca), low calcium with inulin (LcaInu), high calcium (Hca), and high calcium with inulin (HcaInu). The low-calcium diets reflect the compositions of the low daily calcium intake in many countries throughout Asia, Africa, and South America (24, 45). The high-calcium diet reflects the upper boundaries of habitual dietary calcium intake in populations that consume an elevated amount of dairy products typical for northern Europe (24, 45).

### TABLE 2 Composition of experimental diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>Composition (g/kg)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lca</td>
</tr>
<tr>
<td>Acid casein</td>
<td>200</td>
</tr>
<tr>
<td>Corn starch</td>
<td>245</td>
</tr>
<tr>
<td>Dextrose</td>
<td>245</td>
</tr>
<tr>
<td>Palm oil</td>
<td>160</td>
</tr>
<tr>
<td>Corn oil</td>
<td>40</td>
</tr>
<tr>
<td>Cellulose</td>
<td>60</td>
</tr>
<tr>
<td>Inulin (Orafti GR)</td>
<td>0</td>
</tr>
<tr>
<td>Vitamin mix*</td>
<td>10</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>35</td>
</tr>
<tr>
<td>CaHPO$_4$</td>
<td>4.08</td>
</tr>
</tbody>
</table>

| Total                 | 1,000| 1,000  | 1,000| 1,000  |

*Lca, low calcium; LcaInu, low calcium, inulin; Hca, high calcium; HcaInu, high calcium, inulin.

The composition of both the vitamin and the mineral mixtures is according to the recommendations of the American Institute of Nutrition 1993 (66), except that calcium was omitted. In addition, tripotassium citrate was added instead of KH$_2$PO$_4$ and choline chloride was added instead of choline tartrate.

To acquire 40 g inulin/kg, the amount of inulin was increased since Orafti GR inulin contains approximately 10% glucose, fructose, and sucrose.

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**FIG 7** Experimental setup of animal trials. (A) Animal trial with diets supplemented with cellulose or inulin with high or low calcium. After 14 days of acclimatization to the experimental diets, all rats receive a single gavage containing approximately $8 \times 10^9$ CFU of \textit{L. plantarum} Lp900-R. Fecal pellets are collected daily up to 9 days postgavage, and the rats receive a second gavage containing approximately $6 \times 10^9$ CFU of \textit{L. plantarum} Lp900-R to assess local persistence in different intestinal regions (ileum and colon) after 4, 5, or 24 h. (B) Animal trials with diets supplemented with high or low calcium. After 14 days of acclimatization to the experimental diets, half (n = 8) of each diet group receives a single probiotic gavage containing approximately $9 \times 10^9$ CFU of \textit{L. plantarum} Lp900-R or a single synbiotic gavage containing $9 \times 10^9$ CFU of \textit{L. plantarum} Lp900-R suspended in 1 ml of inulin solution (27.5% [wt/vol]). Fecal pellets are collected daily up to 9 days postgavage, and then the rats receive a second gavage (analogous to the initial gavage) containing approximately $6 \times 10^9$ CFU of \textit{L. plantarum} Lp900-R to assess local persistence in different intestinal regions (ileum and colon) after 4, 5, or 24 h.
Animals were acclimatized to the housing conditions and their group-specific diet for 14 days. Subsequently, each rat was intragastrically gavaged with a dose of 1 ml of PBS containing approximately $8 \times 10^9$ CFU of *L. plantarum* Lp900-R, and the intestinal persistence of this strain was monitored during the subsequent 9 days (see below). Rats received a second gavage 10 days after the first gavage of approximately $6 \times 10^9$ CFU of *L. plantarum* Lp900-R. After the second gavage, each dietary group was divided into two separate groups ($n = 4$) that were sacrificed 4.5 and 24 h postgavage, respectively. Distal-ileum and midcolonic luminal contents were collected from the sacrificed animals. Two rats of the Hcalu group and one rat from the Lcalu groups were erroneously euthanized too early (i.e., 3.5 h rather than the intended 4.5 h postgavage) and were therefore not included in the analysis.

To evaluate the persistence of *L. plantarum* Lp900-R when administered as a probiotic or as a synbiotic gavage in a dietary calcium context (Fig. 7B), animals ($n = 16$ per diet group) were fed the Hca or Lca diet (see above; Table 2). After 14 days of acclimatization of the animals to the housing conditions and their diet, the Lca and Hca diet groups were split into groups of eight animals that were intragastrically gavaged with either a probiotic (1 ml of PBS containing approximately $9 \times 10^9$ CFU of *L. plantarum* Lp900-R; designated LcnP or HcnP groups) or a synbiotic suspension (1 ml of PBS containing approximately $9 \times 10^9$ CFU of *L. plantarum* Lp900-R and 27.5% (wt/vol) Orafti GR inulin; designated LcAS or HcAS groups). Subsequently, the intestinal persistence of Lp900-R was monitored during 9 days (see below). Rats received an identical (probiotic versus synbiotic) second gavage 10 days after the first gavage containing approximately $6 \times 10^9$ CFU of *L. plantarum* Lp900-R. After the second gavage, each dietary group was divided into two separate groups ($n = 4$) that were sacrificed 4.5 and 24 h postgavage, respectively, to collect luminal contents from the distal-ileum and midcolon regions.

**L. plantarum** Lp900-R enumeration in fecal samples and persistence data modeling. Fresh fecal pellets were collected at baseline prior to *L. plantarum* administration and daily post-gavage for 9 days. Occasionally, some rats did not deliver a fecal pellet. Fecal pellets were weighed and homogenized after the addition of a fixed volume of PBS (1 or 2 ml) using a T10 basic Ultra-Turrax (IKA-Werke, Staufen im Breisgau, Germany). Fecal slurries were divided into aliquots (four aliquots of 250 μl), one of which was immediately used for enumeration of *L. plantarum* strain Lp900-R CFU, whereas the other aliquots were stored at ~80°C for later analyses (see below). Weighed luminal content samples from the colon and ileum were also homogenized after the addition of a fixed volume of PBS (1 or 2 ml) and subsequently processed in the same way as the fecal samples. For *L. plantarum* enumeration, fecal slurries were shortly centrifuged (3,000 × g, room temperature, 5 s) to remove fecal debris, and serial 5-fold dilutions in PBS were plated on MRS agar supplemented with 50 μg/ml rifampin, followed by incubation at 37°C for 2 days. The selective enumeration of strain Lp900-R was verified by plating fecal baseline samples with or without a spiked-in population of approximately $1 \times 10^6$ CFU of Lp900-R. The lower detection limit was estimated at $4 \times 10^3$ CFU/g of fecal wet weight, which was subsequently used as a fixed limit of detection for all samples in which no Lp900-R colonies were recovered. Dry-wet weight ratios were determined by freeze-drying collected fecal aliquots to evaluate the effect of inulin and calcium on the water content of the fecal material.

To estimate the persistence kinetics of Lp900-R in individual rats, the recovered CFU numbers on consecutive days postgavage per rat were used to model the microbial density decline rate, using Weibull model (56) fitting in R v3.6.1 via the Levenberg-Marquardt nonlinear least-squares algorithm (minpack.lm v1.2-1) (67). The CFU recovery on day 1 was used as a fixed initial number of microorganisms, N(0), for each rat. The Weibull model fitting parameter $\beta$ was employed to decide whether a linear model accurately described the data, using a 95% confidence interval ($\beta$ values that include 1 are considered linear). Most of the data (~90%) were assessed to be linear, and manual inspection of models that were considered as nonlinear revealed that such models either overestimated the initial decline rate (day 1 postgavage) or were unrealistically fitted (i.e., an inclining curve) that may be due to limited data points and inherent noise in the CFU recovery in these experiments. Therefore, a linear model was employed to estimate the *L. plantarum* abundance-decline rate in individual animals to reliably compare samples within and between the experimental groups. Animals were excluded if there were fewer than three data points available (i.e., 1 rat in the Hca group and 2 in the Lca group) or when the calculated root mean square error (RMSE) of the observed versus model-predicted residuals exceeded twice the median within the distribution of the calculated RMSE (i.e., 1 rat in the Lca group, 2 rats in the HcAP group, and 1 rat in the HcAS group) (see Fig. S1 in the supplementary material).

**Statistical analysis.** Shapiro-Wilk normality tests were performed on all data sets with a confidence interval of 95%. Significant differences in growth parameters (i.e., $OD_{600}$ or pH) for more than two groups were detected by a one-way analysis of variance (ANOVA) with post hoc Tukey’s multiple-comparison tests with $\alpha = 0.05$, and for comparisons between two groups with a standard two-sided unpaired Student t tests with $\alpha = 0.05$, using GraphPad Prism version 8.00 for Windows (GraphPad Software, San Diego, CA). The results of persistence curves over time are expressed as log$_{10}$, converted means ± the standard errors of the mean (SEM). Significant differences between intestinal persistence and linear declines rates in the different groups of rats (diet or mode of administration differences within dietary calcium context) were detected by a two-sided nonparametric Mann-Whitney U test using GraphPad Prism version 8.00 for Windows. Significant differences between dry/wet ratios for the collected rat fecal material were assessed by unpaired two-sided t tests with $\alpha = 0.05$ using GraphPad Prism version 8.00 for Windows.

**Data availability.** The genome sequence information of *L. plantarum* Lp900 is available from the NCBI (accession numbers CP059168 to CP059174) (Fuhren et al., unpublished).
SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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Inulin increases L. plantarum Lp900 persistence by altering host microbiome composition and modulating host immune response. Copyright © 2021 American Society for Microbiology. All Rights Reserved.


